Supplementary Information

Supplementary Methods

Whole mount in situ hybridization

In situ hybridization was modified (Leung et al, 2003) In brief, embryos were hybridized at 68^{o}C overnight with antisense probe in hybridization buffer (Hyb), then washed at 68^{o}C by 66% Hyb/ 33% 2x SSC for 30 minutes, 33% Hyb/ 66% 2x SSC for 30 minutes, 2x SSC for 15 minutes and 0.2x SSC for 1 hour. After colour staining with NBT/BCIP (Roche, Indianapolis, IN), the embryos were washed in 100% ethanol for 1 hour. Zebrafish embryos were documented using an MZFL3 stereomicroscope (Leica Microsystems, Wetzlar, Germany) equipped with a Plan-Apo 10X/0.125 NA objective lens, a DEI-750D CE digital video camera (Optronics, Goleta, CA), and ImagePro+ version 4.1 software (Media Cybernetics, Silver Spring, MD).

RT-PCR

Trizol (Invitrogen, Carlsbad, CA) was used to isolate total RNA from wildtype embryos from different developmental stages as indicated. After treatment with RQ1 DNase, 1 μg RNA was reverse-transcribed using Moloney murine leukemia virus (MMLV) Reverse Transcriptase (RT; Promega, Madison, WI). Polymerase chain reaction (PCR) of the cDNA using Titanium Taq (BD Biosciences, San Jose, CA) for 22 to 30 cycles with *gpr161* primers and actin primers, respectively: *gpr161* primers (forward: 5'-CCTACTTGCTAACACCCAGCAACAAG and

reverse: 5'-GGCCACCAGCGGCAAAAGGCAACACC); and

actin control primers (forward: 5'-CCCCGTGCTGTCTTCCCATCCATCGTGGG and

reverse: 5'-CAACACGCAGCTCGTTGTAGAAGGTGTG).

Detection of MO blocking function by in vitro system

The efficacy and sequence specificity of the MO were assessed using an in vitro transcription and translation coupled system (TNT Coupled Reticulocyte Lysate Systems, Promega, Madison, WI). The *gpr161* PCR fragment was cloned into BamHI/XhoI sites in

pcDNA3.1/V5-His-TOPO (Invitrogen, Carlsbad, CA). 0.5 μg of *gpr161* cDNA was used as template and different MO oligos were added. Western blotting was performed using anti-V5 antibody (Invitrogen, Carlsbad, CA). The immunoreactive proteins were detected by enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ). Anti-V5 antibody was used to detect the in vitro translation of Gpr161-V5 fusion protein and band intensity was quantitated and presented in a graph as % of translation compared to control using LumiAnalyst software and LumiImager system (Roche Diagnostics/Boehringer Mannheim Corp., Indianapolis, IN). PCR primers for the *gpr161*-V5 fusion construct were:

*gpr161*V5-F2: 5'-GGATCCCCAGTCTGGAGAAAGTTACTCCTGCTG

*gpr161*V5-R1: 5'-CTCGAGAACATTTTTTCCTCGCGCTCCATTTC

Quantification of cardiac looping, lefty2, spaw and bmp4 expression, and visceral asymmetry

Using *cmlc2* in situ marker for the cardiac chambers, we can determine the cardiac looping phenotype. Normal looping of the ventricle to the right (D-looping) was calculated as percentage of total number of embryos (n) in each experiment. Normal *lefty2 and spaw* expression were detected on the left lateral plate mesoderm and was calculated as percentage of total number of embryos (n) in each experiment. Normal *bmp4* expression with left biased expression in the cardiac cone and heart tube was calculated as percentage of total number of embryos (n) in each experiment. *foxa3* marks the developing gut primordium, liver and pancreatic buds. Normal visceral asymmetry exhibits liver on the left and pancreas on the right and was calculated as percentage of total number of embryos (n) in each experiment. All graphical values were expressed as average $\pm$ S.E.M. (standard error of the mean) from more than 3 injection experiments as indicated in the figure legends. Standard t-test was used to determine if there is significant different between the *gpr161* knockdown and control groups. For rescue experiment, the t-test was used to determine if RNA rescue was significant different from the *gpr161* knockdown alone. Significant difference were indicated on the graphs with $p<0.01$. All p-values were calculated using a 2-tailed t-test.

Histogram analysis and mathematical modeling of Ca^{2+} signal

Each individual fluorescent image of Ca^{2+} signal in gray scale (8-bit) was analyzed by histogram analysis using the ImageJ software (NIH). A total of 18 individual embryos in each group were combined into a single distribution curve, then use mathematical modeling to predict the fraction of normal, intermediate and high level Ca^{2+} signal in each group (Supplementary Fig. 7). The result was based on the best fit value of correlation coefficients of 0.997 in control, 0.995 in *gpr161* knockdown, 0.993 in *ncx1*-RNA rescue and 0.979 in *pmca*-RNA rescue experiments, respectively.

Supplementary Figure Legends

Supplementary Fig. 1. Transmembrane prediction of zebrafish Gpr161 protein using the Hidden Markov Model. The predicted transmembrane region (red), extracellular (pink) and intracellular (blue) loops were indicated (A,B) and the transmembrane region of the zebrafish Gpr161 protein sequence was shown in bold, italic and boxed (C).

Supplementary Fig. 2. Phylogram and RT-PCR analysis of the zebrafish *gpr161*. (A) Phylogram analysis revealed zebrafish Gpr161 protein is highly similar to the human counterpart within the purine receptor cluster branch of the δ-group of RHODOPSIN receptors. The protein sequences were aligned using T-coffee (http://igs-server.cnrs-mrs.fr/Tcoffee/tcoffee_cgi/index.cgi) and the phylogenetic tree was constructed using the Treetop program with a bootstrap value of 100 (http://www.genebee.msu.su/services/phtree_reduced.html). Genbank accession numbers for human C3AR (NP_004045), human GPR103 (NP_937822), human TRHR (NP_003292), human P2RY11 (NM_002566), human GPR82 (NP_543007), human GPR18 (CAI14306), human EBI2 (P32249), human PTAFR (NP_000943), human GPR101 (NP_473362), human GPR161 (CAI22623), human RGR (AAI01111) and zebrafish Gpr161 (EU090912). (B) RT-PCR detection of the maternal zebrafish *gpr161* transcript at 1 cell stage and zygotic transcript at sphere stage (4 hpf) onward to 96 hpf, for 26 and 30 PCR cycles. *actin* RT-PCR was used as RNA loading control,

for 22 PCR cycles. (C) *gpr161* expression in 1dpf to 3 dpf zebrafish embryos. (D) Negative control of using sense probe of *gpr161*. All scale bars were 100μm.

Supplementary Fig. 3. Translational blocking by *gpr161*-MO morpholino detected by in vitro cell lysate assay. (A) Two translational blocking morpholinos, targeted against the 5'UTR upstream of the ATG start codon of zebrafish *gpr161* open reading frame, which was fused to V5-6xHistidine epitope. (B,C) Dose dependent inhibition of Gpr161 translation by two independent *gpr161* morpholinos (*gpr161*-MO#36 and *gpr161*-MO#24) using in vitro cell lysate. No inhibition by 5-base mismatch control morpholino (mismatch-MO#28). Anti-V5 antibody was used to detect the in vitro translation (B) of Gpr161-V5 fusion protein and band intensity (C) was quantitated as % translation compared to control in the graph. No morpholino as control (100%), 10μM MO#24 (7%), 1μM MO#24 (56%), 0.1μM MO#24 (84%), 10μM MO#28 (79%), 1μM MO#28 (92%), 0.1μM MO#28 (79%), 10μM MO#36 (0%), 1μM MO#36 (13%) and 0.1μM MO#36 (40%). (D) Both *gpr161* morpholinos (MO#24 and MO#36) gave similar cardiac looping defect. All scale bars were 100μm.

Supplementary Fig. 4. (A) *nkx2.5* expression in control and *gpr161* knockdown embryos. (B) Confocal imaging of microcilia in Kupffer's vesicle. Anti-tubulin staining (red) of microcilia of control and *gpr161* knockdown embryos in the Kupffer's vesicle at 14 hpf. All scale bars were 100μm.

Supplementary Fig. 5. Ca^{2+} green imaging of intracellular Ca^{2+} in zebrafish embryos. Pseudocolour of fluorescent images of Ca^{2+} signal in posterior mesoderm near the Kupffer's vesicle of control, *gpr161* knockdown, *ncx1*-RNA rescue and *pmca*-RNA rescue of *gpr161* knockdown embryos at 14 hpf. Each group contains 18 different embryos organized as decreasing order of Ca^{2+} green intensity. All scale bars were 100μm.

Supplementary Fig. 6. Histogram analysis of Ca^{2+} green signal in a representative embryo in control, *gpr161* knockdown, *ncx1*-RNA rescue and *pmca*-RNA rescue of *gpr161* knockdown embryos.

Supplementary Fig. 7. Mathematical modeling of combined histograms in each group to predict the fraction of normal, intermediate and high level Ca^{2+} signal.

Supplementary Table 1. Effect of *gpr161* knockdown and RNA rescue on cardiac looping.

Supplementary Table 2. Effect of *gpr161* knockdown on *lefty2* expression on lateral plate mesoderm.

Supplementary Table 3. Effect of *gpr161* knockdown on *spaw* expression on lateral plate mesoderm.

Supplementary Table 4. Effect of *gpr161* knockdown on left-biased expression of *bmp4*.

Supplementary Table 5. Effect of *gpr161* knockdown on visceral asymmetry marked by *foxa3*.

Supplementary Table 6. Effect of *gpr161* knockdown and rescued by *ncx1* and *pmca* RNA on cardiac looping.

Supplementary Reference

Leung, T., Bischof, J., Söll, I., Niessing, D., Zhang, D., Ma, J., Jäckle, H., Driever, W., 2003. *bozozok* directly represses *bmp2b* transcription and mediates the earliest dorsoventral asymmetry of *bmp2b* expression in zebrafish. Development 130, 3639-3649.